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GRANT NUMBER: DAMD17-94-J-4342

TITLE: Role of a Novel Antioncogene that Prevents Metastatic Spread of Disease

PRINCIPAL INVESTIGATOR: Timothy E. Kute, Ph.D.

CONTRACTING ORGANIZATION: Bowman Gray School Medicine of
Wake Forest University
Winston-Salem, NC 27157

REPORT DATE: July 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1996	Annual (1 Jul 95 - 30 Jun 96)

4. TITLE AND SUBTITLE
Role of a Novel Antioncogene that Prevents Metastatic Spread of Disease

5. FUNDING NUMBERS
DAMD17-94-J-4342

6. AUTHOR(S)
Timothy E. Kute, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Bowman Gray School of Medicine of Wake Forest University
Winston-Salem, NC 27157

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The purpose of this grant is to determine a mechanism of action for a novel antioncogene (i.e. nm23) which prevents the metastatic spread of disease.. Our data would indicate that there is no relationship of nm23 expression with proteolytic factors such as cathepsin D, urokinase plasminogen activator, its receptor and its inhibitor. High levels of these factors have been shown to predict metastatic disease in breast cancer patients. Both western blot and ELISA analyses and also by immunohistochemistry were used to prove this lack of correlation. We did show that nm23 expression was related to metastatic potential using cell lines and xenographs. We also performed a clinical trial to determine if nm23 could predict prognosis in node negative breast cancer patients. Our results indicate that nm23 is not a independent prognostic indicator. Our results do indicate that when MDA-MB-231 cells are transfected with nm23 gene, they have a lower motility and become less metastatic in the nude mouse model system.

14. SUBJECT TERMS
Breast Cancer nm23, Proteases, Metastatic Spread of Disease

15. NUMBER OF PAGES
46

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Timothy E Kule

PI - Signature Date

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ATTACHMENTS

INTRODUCTION

Since this is a 2nd year report, most of the background and preliminary data have been described in the first report and will not be repeated here. Since the last report, there was a international symposium in Paris in Oct. 1995 on nm23/ nucleoside diphosphate transferase(1). I was fortunate to attend this meeting and it was well represented by individuals from both the clinical and basic science areas. There was no clear consensus, however, on how nm23 can prevent metastatic spread of disease. A recent abstract by MacDonald *et al* (2) demonstrated that point mutations in at amino acid site 96 and 120 resulted in an nm23 protein that is not able to decrease motility as previously described in a transfected cell line (3). They speculated that the biological function of nm23 is to control motility. Besides the recent review on the clinical utility and function of nm23 (4), a recent paper by Toulas *et. al.* has demonstrated that the nm23-H1 gene product was inversely related to prognosis in breast cancer(5). They speculated that the nm23-H2 is not a prognostic marker in breast cancer. This has been previously demonstrated but it is now confirmed. It should also be noted that the relationship between nm23 expression and prognosis in breast cancer and other cancers is very controversial (4).

The goal of this project was to determine if nm23 could down regulate the expression of proteolytic activity in tumor cells and therefore prevent the cells from escaping the primary site. In order to determine this, three aims were initially proposed.

1. TO CORRELATE NM23 EXPRESSION WITH FACTORS INVOLVED IN PROTEOLYTIC DEGRADATION

2. TO LOCALIZE NM23 EXPRESSION IN RELATIONSHIP TO PROTEOLYTIC FACTORS USING IMMUNOHISTOCHEMISTRY

3. TO DECREASE METASTATIC POTENTIAL BY TRANSFECTION OF NM23 INTO BREAST CANCER CELL LINES CONTAINING A lacZ MARKER GENE

We planned to use human breast cancer tissue for the first two aims and to use two defined breast cancer cell lines for aim 3. If our hypothesis is correct, our results would indicate that as nm23 expression increased there would be a decreased expression of proteolytic factors which have been demonstrated to be indicators of poor prognosis and metastases. These prognostic factors were: cathepsin D (6), urokinase plasminogen

activator (7,-9), its receptor(9), and its inhibitor(7,8). Our second aim was to be able to define these markers by immunohistochemistry as well as ELISA and western blot techniques. It was hoped that the two methods would correlate and add new information concerning the function of nm23. Finally, we want to ask if higher levels of nm23 really did decrease the metastatic potential using a nude mouse model. In this report, we will discuss our results to date and will also indicate what new directions are beng investigated in order to better answer the question as to how nm23 expression could be working in preventing the spread of breast cancer.

BODY

The first annual report for this grant gave a detailed description of the methods and procedures will not be presented here. These procedures include: Western blot, ELISA, immunohistochemistry, synthesis of vectors and transfection of tumor cells and use of the athymic nude mouse model system. Other procedures that were not part of the grant or have been improved will be discussed in the context of the results.

Negative comments from the first review will be addressed at the start of this report.

Technical issue #1 *" The immunohistochemical detection assay for the nm23 protein uses human breast carcinoma as a positive control"*
Question: Would this not be a good positive control for these tumors?
Comment: We initially used the formalin fixed and paraffin-embedded MCF-7 cell blocks as our positive control and determined the correct dilutions for the analysis. We have now examined a large series of patients and determined the % tumor postive in these cases. Since we are measuring relative values, it was felt that the staining should be compared with each patient and we used MCF-7 as a test sample

Technical issue #2: *"Semiquantitative grading can be very subjective and should be done in a blinded fashiion to guarantee nonbiased results"*
Comment: Dr. Kim Geisinger is the only patholigist involved in this study. He is blinded to any informatin on these patients prior to the analysis.

It should be noted that this grant was written for 4 years but was funded for only 3yrs at one half the cost. The amended statement of work should have been provided for reviewer.

RESULTS BASED ON THE AIMS AND THE STATEMENT OF WORK

AIM #1

The task of aim one was to determine the relationship between nm23 expression and various prognostic factors with proteolytic factors being the most important. We have an accepted abstract on this section which will be presented at the International San Antonio Breast Cancer Symposium in December of this year (see appendix). The results can be summarized in table #1 and table #2.

TABLE 1---CORRELATION OF NM23 TO PROTEOLYTIC FACTORS*

	CAT D (N=88)	uPA (N=111)	PAI-1 (N=111)	uPAR (N=111)
NM23-H1	R=-.04 P=NS	R=+.13 P=NS	R=+.12 P=NS	R= +.25 P= 0.008
NM23-H2	R=+.09 P=NS	R=+.23 P=0.02	R=+.15 P=0.10	R=+.23 P=0.01

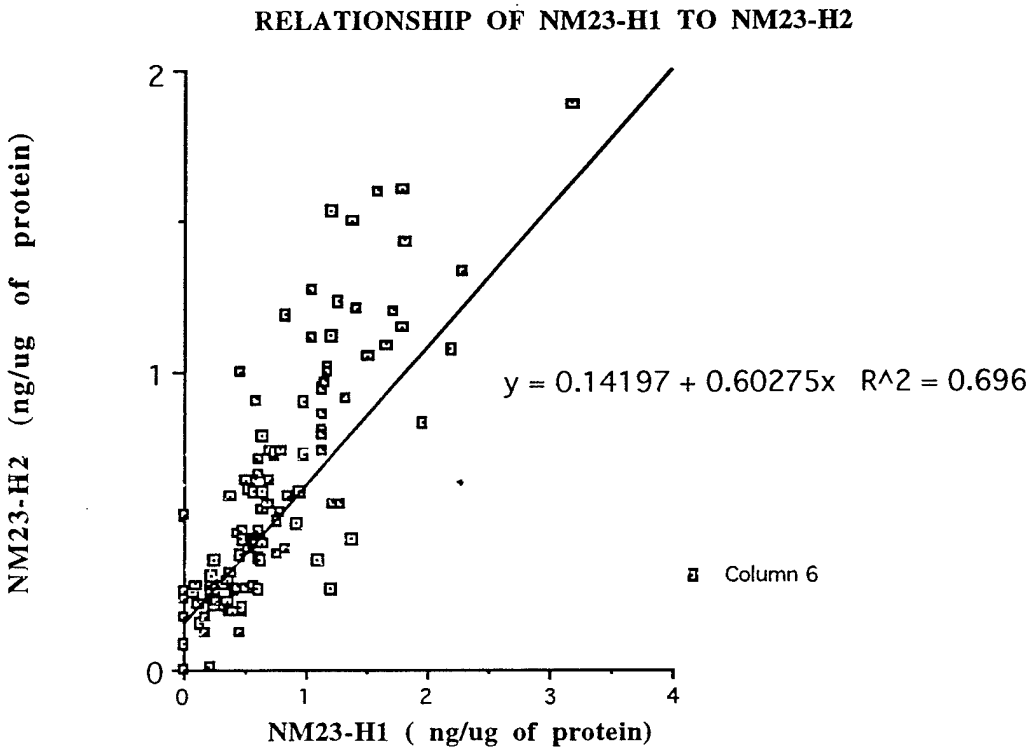
***R is the correlation coefficient and p is the significance where CATD is cathepsin D, uPA is urokinase plasminogen activator, PAI-1 is plsaminogen activator one, and uPAR is the uPA receptor.**

From this data, a strong INVERSE correlation (R value) of nm23 H-1 or nm23 H-2 with any of the proteolytic factors does NOT exist (table 1). One actually observes a SIGNIFICANT positive correlation with nm23-H1 to uPAR (p = 0.008). Secondly, there is a SIGNIFICANT positive correlation with nm23-H2 with uPA and uPAR (p = 0.02 and 0.01 respectively). Based on this preliminary data, one would suggest nm23 does not inhibit metastatic spread of disease by the inhibitiion of the production of proteolytic factors. Since these samples have been analyzed for steroid receptor, ploidy and %S activity, they were correlated with nm23 expression.

TABLE 2 CORRELATION OF NM23 WITH OTHER CLINICAL FACTORS

	ER	PR	PLOIDY	S% PHASE
NM23-H1	NS	NS	P = 0.06	NS
NM23-H2	NS	NS	NS	NS

Our results indicate that steroid receptor status and %S activity were not related to nm23 expression. There was some correlation of nm23-H1 with ploidy. Diploid tumors had a higher nm23-H1 expression compared to the aneuploid tumors. There is data to suggest that aneuploid tumors have a worst prognosis. Our data that aneuploid tumors have a lower nm23-H1 expression than diloid tumors would be in agreement with this prognostic factor. Finally, our results do indicate that nm23-H1 expression is related to nm23-H2 expression (Figure #1)



There are still another 40 samples that need to be compared. These samples will be done during the next year as defined in the grant proposal. We will use this last data set as a confirmation of the previous results.

AIM #2

The second aim of this project is to use immunohistochemistry (IHC) as a method of analysis to determine the relationship between nm23 expression and expression of proteolytic factors. The details for the IHC methodology can be found in the first report and is also available in the paper that has been submitted to Cancer (see attachments). We would like to compare expression of these factors as determined by two different

procedures (IHC vs. Western or ELISA procedures). Secondly, we would like to know if high levels of nm23 are related to low levels of the various proteolytic factors as determined by % tumor cells staining, the intensity of staining, and the localization of staining. List below is a table of the results so far obtained:

DISTRIBUTION OF IMMUNOHISTOCHEMISTY STAINING IN BREAST CANCER PATIENTS

		% TUMOR CELLS STAINED		INTENSITY		
FACTOR	NUMBER	MEAN: MEDIAN	RANGE	RANGE	MEAN	PRIM.SITE
NM23	87	56%: 70%	0 TO 100	0 TO 3+		CYTO
CAT D	79	9%: 0%	0 TO 100	0 TO 3		CYTO
uPAR	77	5.7%: 0%	0 TO 80	0 TO 3		CYTO

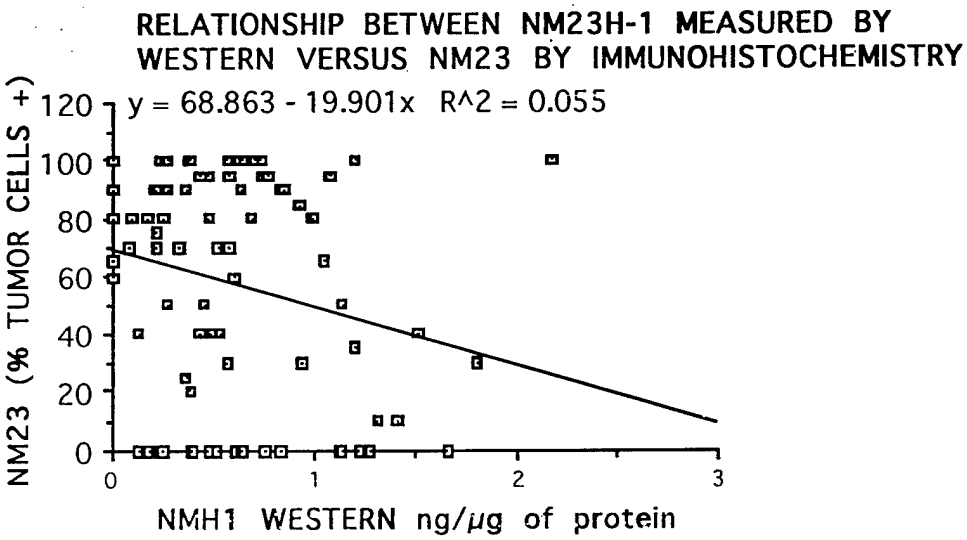
All of the above samples were proven to have tumor and were analyzed by the same person (Dr. Kim Geisinger) in a blinded fashion. Now that we have the procedure down for the above factors, we should be able to complete the study on the remaining patients.

The remaining factors of IHC analyses (i.e. PAI-1 and uPA) have been very difficult to quantify. In the PAI-1 study, the major problem has been the high background binding which is not related to the non-specific binding since our negative controls are very good. We have tried to dilute the primary antibody but loose sensitivity. Several different antibodies have been used but none have been very specific. We have also tried to increase the specific binding by antigen retrieval methods but these procedures have not been successful. As an alternative procedure, we have attempted "in situ" hybridization techniques for the PAI-1 mRNA expression (10). We observed high levels of background staining by the antisense mRNA that were similar to sense mRNA hybridization. Secondly, both sense and antisense hybridization were sensitive to RNAase. Finally, we have attempted to use frozen sections instead of formalin fixed and paraffin embedded tissues. Our results from these studies indicate a high background which is attributed to the endogenous biotin levels in the tissues. This high background can be eliminated using blocking techniques and this hopefully will give us some better data.

In the uPA IHC studies, we have observed very little specific binding with the presently used antibodies from Oncogene Science which have been reported to be useful in formalin fixed and paraffin embedded sections. This is after changes in dilution, antigen retrieval, and other

techniques as described in the PAI-1 studies. We have ordered new antibodies and will continue doing these studies.

Our first analysis of the available data was to determine if quantitative expression by ELISA or western blot procedures can be related to percent of tumor cells staining or intensity of staining. The figure below demonstrates that there is no relationship of percent of tumor cells staining and nm23-H1 expression by Western blot analysis:



Using the intensity of staining for nm23 rather than the % of tumor still needs to be analyzed in more detail. Our data does indicate that staining intensity is related to the percent of tumor cells stained in the tissues. We have yet to define non-tumor staining in relationship to nm23 expression.

Similar comparisons of Cathepsin D and uPA gave the same results (data not shown). These results and the results from nm23 would indicate that IHC expression can not be directly correlated to the values obtained by ELISA or western blot analysis. We have noted that there are non-tumor cells that stain for these factors and have defined the amount. This non-tumor expression could effect the relationship between the methods. Finally, there could be a problem of sampling artifact between the two procedures. In the final year of this study, we will try to address these issues as we define better procedures and have more patients in the data set.

The second issue is to compare nmn23 expression as obtained by IHC to the expression of the other proteolytic factors performed by IHC. Using

percent of tumor cells stained, there was no correlation between nm23 expression and the expression of either cathepsin D or uPAR (data not shown). These data are still inconclusive but are similar to the results found in aim #1 where nm23 expression is NOT related to the listed proteolytic factors.

In conclusion, the immunohistochemistry studies have taken a large percent of our time during the second year. It is clear that one can not compared results from the two different procedures and there is no INVERSE relationship of nm23 expression to proteolytic expression using IHC procedures. We will continue to work on better methods of analysis. Secondly, these patients will be monitored for prognosis in light of the IHC staining and ELISA and western blot results. It is our hope that these assays will be able to predict the clinical outcome of patients with breast cancer.

AIM #3

In Aim 3, the goal was to demonstrate that cells containing high levels of nm23 are less metastatic. The original aim was to do these studies on cell lines only. As discussed in the first annual report, we have measured the metastatic disease in athymic nude mice using the lacZ system (Figure 10 first annual report). We have finished the transfection of the MDA-MB-231 cells and demonstrated an increase in nm23 expression by western blot analysis (Figure 11 first annual report). Since that time, we have transfected a second cell line GI101 and a list of nm23 expression levels for both of these cell lines are given below:

TABLE X-- EXPRESSION OF NM23-H1 IN TWO BREAST CANCER CELLS AND TRANSFECTED CLONES*

	N	MEAN	S.E.
MDA-MB-231	7	0.173	0.047
CLONE 25	6	0.645	0.106
CLONE 40	6	0.590	0.172
CLONE 47	6	1.438	0.250
GI-101	3	0.380	0.026
CLONE 10	3	1.990	0.615
CLONE 15	3	2.350	0.388

*** N is number of assays done, SE is standard error. The mean is ng/ug of protein extract.**

The MDA-MB-231 cell lines and clones have now been tested for metastatic potential using the athymic nude mouse model. The parent cells had a metastatic potential of 71%. The MDA-MB-231 clone 40 and MDA-MB-231 clone 47 had metastatic potentials of 38% and 40% respectively. This data will also be presented at the International San Antonio Breast Cancer Symposium (see Appendix). These data are similar to the results of Leone *et al.* where the cells were MDA-MB-435 (11). We are now in the process of measuring the metastatic potential on the GI-101 cell line. This cell line was developed by Dr. J. Hurst (12) at the Goodwinn Institute and she has demonstrated that this cell line is highly metastatic in the athymic nude mouse model. We have decided to send the parent, clone 10 and clone 15 to this group for the metastatic potential analysis. It was felt that they were more familiar with their cell line and that it would save time and money. I have enclosd a statement from the Goodwin Institute defining their protocol (see appendix). They have just started injecting cells into the animals and should have some results by the fall.

A second way to correlate metastatic potential with nm23 expression is to use human xenographs. We have been working with Dr. R. Mehta who has provided us with solid tumors that were obtained from human tissue implanted into an athymic nude mouse. She has then determined the metastatic potential for these xenographs and we have analyzed these for nm23-H1 expression. Included in this table below are the nm23-H1 expression values for the known cell lines and their metastatic potential from our studies and from Dr. R Metha results.

EXPRESSION OF nm23-H1 IN BREAST CANCER CELL LINES, AND XENOGRAFTS DERIVED FROM PRIMARY HUMAN TUMORS

EXPRESSION OF nm23-H1 (ng/μg of protein)			METASTATIC POTENTIAL	
CELL TYPE	N	MEAN (± SE)	METS/ANIMAL	(%)
MCF7	6	1.642 (0.137)	2/6	(33)
MDA-MB-231	7	0.173 (0.047)	5/7	(71)
MDA-MB-435	5	0.369 (0.118)	5/10	(50)
XENOGRAFT ¶	8			
UISO-BCA-1	8	0.542 (0.076)	0/50	(0)
UISO-BCA-4	8	0.429 (0.172)	0/50	(0)
OSHTMAM-4	7	0.605 (0.086)	1/5	(20)
MAX-F-401	6	0.388 (0.047)	3/8	(38)
MAX-583	8	0.291 (0.074)	1/5	(20)
MAX-713	7	0.182 (0.069)	1/5	(20)
MAX-713 (mtg)§	8	0.137 (0.039)	1/5	(20)
GI-101	9	0.258 (0.038)	3/5	(60)

¶ Human breast cancer specimens were transfered to nude mice. The resulting tumor, grown in nude mice was analyzed for nm23 -H1 by western blot.
§ Xenograft was injected in matrigel (mtg).

The relationship of nm23 expression with metastatic potential is inversely correlated (R= -0.51). Although this does not reach the significance level (p = 0.19), it does agree with the proposal that higher levels of nm23 are less metastatic. Further analysis using more cell lines will be necessary to confirm this negative correlation. However, it does demonstrate that high levels of nm23-H1 are related to lower metastatic potential.

The third way to determine if nm23 expression is related to metastatic spread of disease is do perform a clinical study. The details of this clinical study are given in a manuscript that has been submitted to Cancer(13). Patients were selected who were node negative and on whom clinical follow-up is known. We have selected 19 patients without recurrence and 21 patients with known recurrences. We have attempted to match these patients so that we could better understand the impact of nm23 expression on recurrence of disease (see tables of submitted paper). Using immunohistochemisty procedures, we have defined as nm23 positive, all tumors that contain > 40% positive tumor cell staining. The Kaplin Meier analysis indicates that there is no significant difference between these two groups (see figure 3 of submitted paper). Use of other cut point analyse did not change the observed findings. Although there are few patients in the groups, this study defines the prognostic potential of nm23 in node negative breast cancer patients which is independent of

other clinical factors. The reasons why these results do not agree with the current literature is unknown but could involve methodology differences. Our antibody used in this study measures both nm23-H1 and nm23-H2. There are some reports that would indicate that only the nm23-H1 expression is important in prognosis (5). There are other studies, however, using other polyclonal antibodies that have shown prognostic importance for nm23 (4). Secondly, our data would suggest that nm23-H1 is highly correlated with nm23-H2 (see figure 1 above). It is also possible that nm23 is not an independent prosnostic marker and matching patients as done in this study has resulted in demonstrating that nm23 expression does not predict prognosis.

FUTURE DIRECTIONS

Since our data would suggests that nm23 does not work through regulation of proteolytic factors and steroid receptors, and is not related to proliferation, the question would be, how does it work? I was able to attend the First International nm23/NDPK conference and met Dr. Jason Kantor. He has published data in the relationship of nm23 to motility (3). We sent him our MDA-MB-231 parental cell line and three clones. He then provided us with the following data:

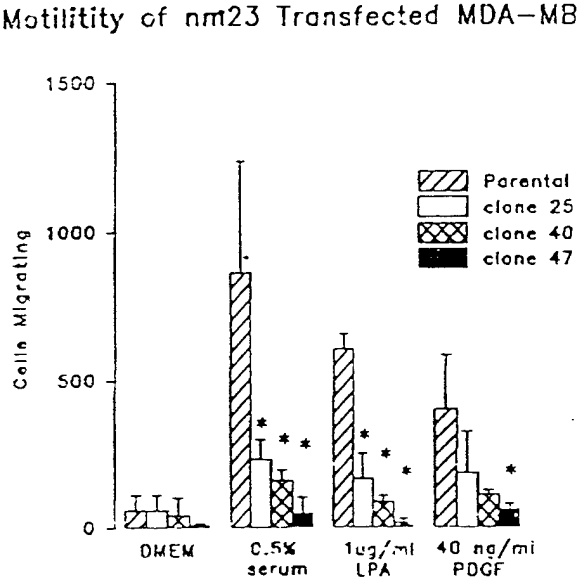


FIGURE 1

Motility studies were performed by adding 250,000 cells to the upper well in a modified Boyden chamber. The bottom well was filled with DMEM containing 0.5% bovine calf serum, 1 µg/ml of LPA, or 40 ng/ml PDGF. The top and bottom wells were separated by a 8µm filter, precoated with fibronectin. After 5 hours, cells were removed from the top of the filter, fixed, stained and counted. All points are the means of three replicates. * Denotes significant difference from parental cell line.

One observes that none of the cells migrate unless they are stimulated with serum, lysophosphotidic acid (LPA), or platlet derived growth factor (PDGF). Low motility of the MDA-MB-231 clones is directly correlated with the high expression of nm23 (See table X). We are now in the process of trying to understand how nm23 might effect motility. A "IDEA" Army grant was submitted to study interesting findings

PROJECTS FOR THE THIRD YEAR

We will finish up the remaining assays for nm23 and the other components as defined in aim 1. There are 40 more samples to be done and we already have the tissues. In Aim 2, we will perform the remaining assays for CAT D, uPAR, and nm23. The immunohistochemical analysis for PAI-1 and uPA still need to be worked on as discussed above. We are hoping that these methodologies can be worked out. In the third aim, we should have data on the metastatic potential of GI-101 with respect to the parental and transfected clones. This will give us time to finalize the data and to present it at the Army meeting and publish these results.

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ABSTRACT
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19th annual san antonio BREAST CANCER symposium

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EXPRESSION OF NM23 IN BREAST CANCER AND ITS RELATIONSHIP TO OTHER PROGNOSTIC MARKERS. Kute TE, Russell RE, Pedersen A, Long R, Zbieranski N, Brunner N, Shelton B

Levels of nm23 have shown an inverse correlation to metastatic potential and poor prognosis in breast cancer patients.The mechanism by which nm23 exhibits this antimetastatic effect is not known. We have measured the expression of the two isoforms of nm23 (H1 and H2) in 117 patients using a quantitative Western blot analysis. We have also measured 8 other reported prognostic markers on the same patients. Steroid receptors were measured by both the biochemical and ICA procedures. Ploidy and cell kinetics were measured by flow cytomety. The urokinase plasminogen activator components were measured by ELISA and the Cathepsin D was measured by an RIA procedure.

Nm23-H1 was directly correlated with nm23-H2 levels (R= 0.84, p = 0.0001) and uPAR (R= 0.25, p=0.008). Patients whose tumors were diploid had significantly higher mean nm23-H1 values than those patients whose tumors were aneuploid (p = 0.06). The expression of nm23-H2 was correlated with uPA, and uPAR (p = 0.02, 0.01, respectively). Neither isoform of nm23 was related to steroid receptors, cell cycle kinetic values or cathepsin D. In multivariate analysis the best correlation of nm23-H1 expression was with uPAR levels (p = 0.003).

SUMMARY: Since proteases and cell proliferation are essential for the metastatic spread of disease, it was proposed that nm23 might be inversely proportional to these factors. Although no correlation was observed between nm23 and protease activity or cell proliferation, it is possible that nm23 may be related to cell motility since this is another factor that regulates the metastatic potential of tumors.

Supported by U.S. Army Breast Cancer Grant (DAMP17-94-J-4342)

ABSTRACTS MUST BE RECEIVED BY: JUNE 1, 1996

Abstract to be Presented by

TIMOTHY E KUTE
First Name MI Family Name

Complete Mailing Address

DEPARTMENT OF PATHOLOGY
MEDICAL CENTER BLVD
WINSTON-SALEM NC
27157-1073

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THE TRANSFECTION OF NM23 INTO THE HUMAN BREAST CANCER CELL LINE MDA-MB-231, RESULTS IN REDUCED METASTATIC POTENTIAL IN NUDE MICE AND A REDUCED RESPONSE TO MOTILITY-STIMULATING FACTORS. Russell, RL, Kantor, J, Geisinger, KR and Kute, TE. The Bowman Gray School of Medicine, Departement of Pathology, Winston Salem, N.C. 27157. Children's Hospital, Boston, MA, 02115.

The antimetastatic gene, nm23 was transfected into the human breast cancer cell line MDA-MB-231. Between 4 and 8 fold induction of nm23-H1 protein was observed in clones MDA-MB-231-40 and MDA-MB-231-47, respectively, as compared to the parent cell line. Analyses of the phenotypic changes associated with the overexpression of nm23-H1 protein were performed in both *in vitro* and *in vivo* experimental systems. The overexpression of nm23-H1 was not associated with alterations in proliferation *in vitro* or in the growth rate of solid tumors in nude mice. The nm23-H1 overexpressing clones were injected into nude mice and analyzed for metastatic potential as compared to the parental cell line. The invasive potential was characterized as the appearance of metastatic lesions in the lungs or peritoneal cavity of nude mice. Whereas the parental cell line MDA-MB-231 produced metastatic disease in 71% of the animals, the transfected clones produced metastatic lesions in 38% (MDA-MB-231-40) and 40% (MDA-MB-231-47) of the animals.

In vitro motility assays were performed on the MDA-MB-231 parent cell line and the two nm23-H1 overexpressing clones, using a modified Boyden Chamber assay. These experiments demonstrated that the MDA-MB-231-47 clone had a reduced response to motility-stimulating factors (PDGF, 0.5% serum, and LPA) as compared to the parental cell line. Additionally, the MDA-MB-231-40 clone which expressed intermediate levels of nm23-H1 protein, exhibited an intermediate reduction in motility as compared to the parental cell line and the high expressing clone. The reduction in motility was statistically significant in the MDA-MB-231-47 high nm23-H1 expressing clone.

In summary, the transfection of MDA-MB-231 breast cancer cell line with the nm-23 gene, resulted in reduced metastatic disease in nude mice and a reduced response to motility-stimulating factors *in vitro*. No other phenotypic difference in growth rate was observed.

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GOODWIN INSTITUTE *for* CANCER RESEARCH, INC.
1850 Northwest 69th Avenue • Plantation, Florida 33313 • Phone: 305/ 587-9020 • Fax: 305/ 587-6378

July 30, 1996

Dr. Tim Kute
Associate Professor of Pathology
Bowman Gray School of Medicine
Medical Center Blvd.
Winston-Salem, NC 27157-1072

Dear Tim,

This letter is intended to state that the Goodwin Institute for Cancer Research, Inc. (GICR) will conduct a collaborative project studying the nm23 gene *in vivo* in our GI-101-A HMT cell line (metastatic breast tumor)

The purpose will be to transfect GI 101-A HMT with clones of nm23 genome and compare two (2) of them with the mock transfected original cell line for differences in tumorigenicity and lung metastasis following injection and growth in athymic nude mice.

Athymic nude female mice (Ncr), GICR stock, 12-14 weeks of age will be used. Three (3) groups of fifteen (15) mice each will be used including several mice to be injected with the original GI-101A line maintained at GICR. The mice are housed and maintained according to ILAR guidelines and frequently monitored for pathogens.

The original cell line is an adherent, non-fastidious tumor with a doubling time of approximately 48 hours. It is grown in 37 degrees C at 5% CO_2. GICR originated the cell line from the GI-101 HMT xenograft line. The original tumor was an infiltrating ductal adenocarcinoma (stage IIIa, T3N2MX). The xenograft has been grown at GICR for over 10 years. The cell line was established in 1994. The nm23 clones were produced by Dr. T. Kute after receipt of the original cell line from GICR (passage 21). Three (30) clones will be injected: GI-101 negative (mock transfected), clone 10 and clone 15.

The passaged cells will be grown in T150 flasks in standard tissue culture media. Cells will be removed with trypsin-EDTA (Sigma), washed, and counted. They will be resuspended in PBS for injection at 2×10^6 per mouse. Mice will not be staged but will be grouped according to approximate size, unless otherwise noted, in cages of 5 mice each. They will be ear notched and individually monitored. We will inject three (3) groups of 15 mice/group, one for each clone. The tumor take and growth rate will be monitored by measuring tumors and weighing mice once weekly. Measurements will be performed with calipers, 0.5 (L x W x H). Growth curves will be generated by Shula Raney on Lotus 123.

At approximately 1500-2000 cu mm (generally a 2-3 month period), the mice will be sacrificed, and prepared for histology. Tumor slices and lungs will be taken and fixed in Holland's fixative (or 10% buffered formalin). Fixing and cutting can be performed by a subcontractor to GICR or at Bowman Gray, if preferred. Reading of slides can be performed at GICR by Drs. Jo Hurst or Keith Wallace or at Bowman Gray. Tumors will be quickly scanned for percent differentiation. Lungs will be viewed and scored for lung metastatic foci of GI-101-A cells by relative count and comparison of experimental and control groups.

Analysis will be performed by Dr. Tim Kute in collaboration with GICR personnel.

Our initial budget will recover our up front costs. This includes purchasing 45 nude mice at $16.00/mouse for $720. Tissue culture supplies will come to $250. Total budget for mice and supplies will be **$970**.

Subcontracting for histology or slide reading will be determined at a later date. Current processing by a subcontractor is $5 / slide. There are generally 2 slides per mouse sample: tumor slice and lung slice. This would amount to another $450. Reading of slides by Drs. Hurst or Wallace would cost $5 per slide x 90 slides or $450.

All or any part of this extra charge may be applied to the study. No charges will be included for GICR labor at this time.

Sincerely,

Michael J. Dauphinée, Ph.D.
Director, GICR

nm23: RELATIONSHIP TO THE METASTATIC POTENTIAL OF BREAST CANCER CELL LINES, PRIMARY HUMAN XENOGRAFTS AND NODE NEGATIVE BREAST CANCER PATIENTS

Running Title: nm23: Relationship to metastatic potential

Rosalind Lee Russell PhD[1], , Kim Robert Geisinger MD[1], Rajeshwari R Mehta PhD[2], Wain White MD[1], Brent Shelton[3] and Timothy Edward Kute PhD[1]

1. Department of Pathology, The Bowman Gray School of Medicine, Medical Center Boulevard, Winston-Salem, N.C., 27157.

2. Department of Surgical Oncology, University of Illinois at Chicago, 840 South Wood Street, 60612.

3. Department of Public Health Sciences Section on Biostatistics, The Bowman Gray School of Medicine, Medical Center Boulevard, Winston-Salem, N.C., 27157.

Supported by The U.S. Army Grant (DAMD17-94-J-4342)

Address for Reprints:
Timothy E. Kute, Associate Professor, Department of Pathology, The Bowman Gray School of Medicine, Medical Center Boulevard, Winston-Salem, N.C., 27157. Telephone (910)-716-2950 Fax (910)-716-7595

Manuscript contents: text pages (20), tables (3) and illustrations (3).

Précis: Analyses of the levels of nm23 protein was performed in breast tumor cell lines, xenografts and a retrospective node negative breast cancer population using immunohistochemical and/or western blot methods. No significant correlation was observed between the levels of nm23 and the metastatic potential of these breast tumor populations.

ABSTRACT

BACKGROUND. Since the discovery of the nm23 gene (non-metastatic) by Steeg *et al.*, in 1988, a number of tumor cohort studies have shown an inverse relationship between the levels of expression of the nm23-H1 protein and disease aggressiveness and tumor metastatic potential.

METHODS. The relationship between the expression of nm23 protein and the metastatic potential of human breast cancer has been analyzed in cell lines, xenografts, and in a retrospective node negative breast cancer population. The node negative breast cancer study consisted of 40 patients, 19 non-recurrent and 21 recurrent. The 40 patients were matched according to age, cathepsin D, tumor size, % S phase, DNA ploidy, steroid receptor status and tumor grade. Nm23-H1 protein levels in cell lines and xenographs were analyzed quantitatively using western blot analyses and semi-quantitatively in tissue sections using immunocytochemistry. Immunocytochemical analysis of node negative breast tumors was graded as the percent of tumor staining positive for nm23 and the intensity of staining. The metastatic potentials of the cell lines and xenografts were assessed as the ability to form metastatic lesions in nude mice. In the node negative breast cancer patients, the metastatic potential was characterized as the incidence of breast cancer recurrence.

RESULTS. The MCF-7 cell line expressed four and ten fold higher levels of nm23-H1 than the highly metastatic MDA-MB-435 and MDA-MB-231 cells, respectively. Among the xenografts and cell lines, there was an inverse correlation ($R = -0.51$) between nm23-H1 expression and metastatic potential in athymic nude mice. The differences between the levels of nm23-H1 among the metastatic and non-metastatic cell lines and xenografts was not statistically significant. Statistical analyses indicated that neither the intensity nor the percent of tumor staining positive for nm23 expression was correlated to the recurrence

of breast cancer in the node negative patient population which had been matched for other clinical prognostic markers.

CONCLUSION. There was an inverse correlation (R=- 0.51) between the levels of nm23-H1 expression in cell lines and xenografts and the metastatic potential in nude mice. In the retrospective node negative breast cancer population, no clear association was demonstrated between the expression of nm23 and breast cancer recurrence. This observation suggests that other factors including methods of analysis are contributing to, or masking the contribution of nm23 expression and the risk for recurrence in our clinical trial.

Key Words: Breast Cancer, Clinical, Metastasis, Node-negative, nm23, Immunohistochemistry

INTRODUCTION

While the mortality associated with breast cancer is most certainly a result of metastatic disease, a clear understanding of the mechanisms involved in the progression of a primary tumor toward metastasis have not been established. The discovery of the nm23 gene (1), and its subsequent identification as a putative suppressor of metastases (2,3) has elicited a great deal of interest in the mechanisms by which the nm23-H1 protein may suppress the formation of metastatic lesions.

The nm23 gene was first identified in a murine melanoma cell line using differential hybridization techniques to characterize clones having high and low metastatic potential (1). Studies demonstrated that transfection of the nm23-H1 gene into highly metastatic cell lines resulted in significantly fewer metastatic lesions in athymic nude mice (4,5). Several human breast tumor cohort studies have shown that reduced nm23-H1 protein or RNA expression was associated with more aggressive disease (2,6-11). Other human tumor cohort studies have demonstrated a similar inverse correlation between the levels of nm23 and overall survival/stage or metastatic potential of hepatocellular, melanoma, gastric, prostatic and ovarian cancers (reviewed in 12). In both neuroblastoma and pancreatic tumor studies, however, elevated levels of nm23 were associated with the more aggressive disease (reviewed in 12).

Two human nm23 genes have been identified, nm23-H1 (13) and nm23-H2 (14) which encode for distinct proteins that are 88% homologous. nm23-H1 and nm23-H2 are identical to human nucleoside diphosphate kinase (NDPK) A and B, respectively (15). Although this observation attributed a biological activity to nm23, no correlation between NDPK enzymatic activity and the suppression of metastasis has been shown (5,16).

This study was undertaken to analyze breast cancer expression of nm23 protein levels in a variety of different sources including: breast cancer cell lines, primary breast cancer xenografts and a retrospective node negative breast cancer population matched for other significant prognostic markers. The levels of nm23-H1 expression in these sources were correlated to metastatic potential based on athymic nude mouse studies or clinical follow-up of breast cancer patients.

METHODS

HUMAN MAMMARY TUMOR XENOGRAFTS AND CELL LINES

The human mammary cell lines MCF-7, MDA-MB-231 and MDA-MB-435 were grown according to recommended procedures and maintained in log growth phase by passaging 1-2 times per week. Human xenografts were established by repeated passages of human breast tumors into nude mice as has been previously described (17-19). The mammary tumor xenograft GI-101 was developed by J. Hurst at the Goodwin Institute for Cancer Research, Inc. Plantation, Florida (17). Xenografts UISO-BCA-1, and UISO-NMT-BCA-4 were established by Dr. R.R. Mehta (18). Human breast carcinoma xenografts MAXI-401, MAXF-713, MAXF-713 with matrigel and MAXF-583 were originally established by Dr. H.H.Fribig, Frieburg University, Frieburg, Germany (19). The xenograft OHSTMAM-4 was established by D. J. Dykes, Southern Research Institute, Birmingham, AL (18). Tumor extracts were prepared from xenografts and cell pellets using methods as previously described (20).

METASTATIC POTENTIAL ANALYSIS

Athymic nude mice 5 to 6 weeks old were treated with either human tumor homogenates, or tumor cells suspensions as described previously (18). The growth of each tumor was monitored and the animals were sacrificed at the end of the experiment (6-8 weeks). The animals were then dissected to determine whether any metastatic disease was present. Metastatic disease was determined by H&E staining of the lungs and other suspected organs of the animal. Any positive tumor site outside the primary was considered to be a metastasis.

WESTERN BLOT PROCEDURE

Tumor extracts (30 -50 µg/lane) from xenografts or cell line pellets, were boiled in SDS-lysis buffer and analyzed using standard SDS-PAGE and Western

blot procedures. Each gel contained molecular weight markers and purified nm23-H1 protein standards ranging from 2 -100 ng. Nitrocellulose membranes (Hybond-C extra; Amersham Life Sciences, Arlington Heights IL.) containing nm23-H1 standards and tests samples were blocked using a 5% casein-Tris buffer. The blots were incubated 2 hours at room temperature with 0.193 μg/ml polyclonal anti-nm23 antibody. The partially purified polyclonal antibody was a generous gift from Oncologix™ and recognizes nm23-H1 and nm23-H2 proteins in human breast tumor specimens. The polyclonal antibody to nm23 was evaluated and compared to the peptide 11 antibody (4) and both antibodies were shown to have comparable immune-reactivity (data not shown). The blots were then washed and the primary antibody was labeled with 1:2000 dilution of biotinylated donkey-anti-rabbit antibody (Amersham Life Sciences, Arlington Heights IL.). Further washing was followed by the labeling of the biotinylated antibody with 1:2000 dilution of streptavidin horseradish peroxidase enzyme (Amersham Life Sciences, Arlington Heights IL.). Finally, the blots were immersed in ECL™ chemiluminescent reagent (Amersham Life Sciences, Arlington Heights IL.) and the nm23 proteins labeled with HRP were detected on x-ray film.

Quantification of the levels of nm23-H1 (17 kDa) in the tumor extracts was performed using the standard curve of the known quantities of nm23-H1 and scanning laser densitometry. Known quantities of nm23-H1 were plotted against optical density x mm and a standard curve was plotted for each experiment.

IMMUNOHISTOCHEMISTRY FOR nm23

The immunohistochemical analyses of nm23 in breast cancer patients were performed according to standard procedures. Briefly, human breast tumors were fixed in 10% buffered formalin and embedded in paraffin. Paraffin sections (4-5 microns) were cut from each breast tumor specimen, deparaffinized in

xylene and rehydrated in a graded series of ethyl alcohol. The tissues were then washed in distilled water for 2-5 minutes.

The polyclonal antibody concentration was established at 19.3 μg/ml . Tissue specimens were analyzed for nm23-H1 using an automated staining system (Ventana 320 ES™; Ventana Biotek System, Inc. Tuscon, Arizona) and manufacturer recommended reagents necessary for the detection of primary antibody using AEC (3-amino ethylcarbazole) as the chromogen. Pretreatment of the tissues with alkaline protease (protease 2; Ventana Medical Systems, Inc. Tuscon, Arizona) for 8 minutes enhanced the sensitivity of the detection of nm23.

One pathologist (K.R.G.) performed all immunohistochemical analyses without knowledge of patient outcome. The criteria for the analysis of each specimen, included: the cytoplasmic staining intensity of the breast tumor (0-3+), the percentage of tumor that stained at the given intensity and the percentage of tumor in the section. Independent evaluations were performed for intraductal and invasive tumor in each specimen

STATISTICAL ANALYSES

Twenty-one recurrent patients were chosen retrospectively and then 19 matched controls were chosen. Matching was attempted according to age, cathepsin D, tumor size, % S phase, DNA ploidy, steroid receptor status and tumor grade. As is evident in Table 2, the two groups are homogeneous with respect to most of the matched factors with the exception of steroid receptor status where the recurrent group had more steroid receptor positive patients. The two sample t-test was used to compare continuous variables (age, cathepsin D and tumor size), and either Fishers exact test or Pearsons chi-square test was used to compare ordinal variables.

The association between nm23 status (> 40% vs ≤40 %) and recurrence and overall survival was assessed by Kaplan-Meier (21) analysis, in which the log-rank test was used to determine level of significance.

The relationship between nm23-H1 expression and metastatic potential was assessed using Pearson's product moment correlation coefficient. Since the analyses for cell line and xenografts were different, partial correlation coefficients were calculated.

Cox proportional hazards model (22) was used to assess differences in recurrence and overall survival between the two nm23 groups after adjustment for other covariants.

RESULTS

nm23 EXPRESSION IN HUMAN BREAST CANCER CELL LINES AND XENOGRAFTS AND THE CORRELATION OF nm23-H1 CONCENTRATION WITH METASTATIC POTENTIAL

We have evaluated nm23-H1 protein levels in breast cancer cell lines, and nude mouse xenografts of primary human tumors using western blot analyses. Figure 1 shows typical western blots containing unknown samples and standards having known concentrations of nm23. The nm23 values of the unknowns were calculated using a standard curve of the standards contained in each gel. In Figure 1A. Lanes 2-6 contain nm23-H1 standards (10-160 ng), lanes 7 and 9 contain tumor extracts from MDA-MB-435 and MCF-7 cells respectively. Lanes 1, 7 and 9 are blank. The levels of nm23-H1 in the three cell lines vary considerably. MCF-7 cells expressed the highest nm23-H1 levels and contained 4 to 10 times higher levels than either MDA-MB-435 and MDA-MB-231, respectively (Table 1). A broad range of values for nm23-H1 was observed among the eight primary human tumor xenografts (Figure 1B). Lanes 1-5 contain purified nm23-H1 protein and lanes 6-13 represent eight different breast cancer xenografts. Lanes 6 and 7 represent xenografts that are non-metastatic in nude mice and lanes 8-13 represent xenografts that are metastatic in nude mice. We utilized the ability to quantify the levels of nm23 in tumor extracts and cell lines to evaluate the variability of nm23 H1 levels in these tissues. The levels of nm23 $\pm$ standard error were compared to the metastatic potential of cell lines (MCF-7, MDA-MB-231 and MDA-MB-435), and eight human xenografts (Table 1).

The levels of expression of nm23 were correlated to the incidence of metastatic lesions in nude mice (described as the metastatic potential). The differences between the metastatic and non-metastatic tissues and cell lines were not significant ($p=0.19$) although a negative correlation ($R=-0.51$) was observed where there appeared to be a trend toward the non-metastatic tissues expressing higher levels of nm23.

IMMUNOHISTOCHEMICAL ANALYSES OF nm23 AND PROGNOSIS OF NODE NEGATIVE BREAST CANCER PATIENTS

Frequently in the clinical environment, the availability of fresh tumor tissue is limited. We have therefore extended our analyses to include immunohistochemical staining techniques performed on formalin-fixed paraffin-embedded tissues. The polyclonal antibody to nm23 recognizes both nm23-H1 and nm23-H2 by western and immunohistochemical analyses and has reactivity comparable to peptide 11 antibody described by Leone *et. al.* (5) (data not shown).

A node negative breast cancer pilot study was selected as a means of evaluating the effect of nm23 expression on the metastatic potential of a clinical breast cancer population. A node negative population was selected since this is the patient population in which it would be most useful to predict the risk for recurrence. The original patient population from which this sample was drawn has been described in detail (23). At the time of selection of the pilot study group, 26 patients from the original group had suffered a relapse. The recurrent patients were selected and matched with a non-recurrent group of node negative patients from the original population. Criteria that were used to match the recurrent and non-recurrent groups included: age, cathepsin D concentration, tumor size, %

aneuploidy, % S phase, steroid receptor status and tumor grade. The analyses of the recurrent and non-recurrent populations are shown in Table 2. The mean time of follow-up was significantly longer in the non-recurrent group in order to reduce selection bias as a result of short follow-up times. Although there was a trend toward non-recurrent patients expressing lower steroid receptor positivity, this difference was not statistically significant using the Fisher exact test ($p=0.06$ and $p=0.18$ for estrogen and progesterone receptors, respectively). Since steroid receptors have not been proven to be prognostic in the complete clinical trial (23), it was felt that the differences in receptor status compatibility would not be critical. Additionally, since the non-recurrent group had a lower percentage of steroid receptor positivity than the recurrent group, the selective advantage for survival would, if anything, give a benefit to the recurrent group.

Extensive analyses of the literature has shown considerable variability with respect to the methods of grading nm23 expression by immunohistochemical techniques. Nm23 has been shown by others to be related to metastatic potential (reviewed in 12); therefore, we analyzed the invasive and intraductal components of each breast cancer specimen independently. Since the staining intensity is a variable that is highly subjective and can have considerable variability within and between experiments, nm23 positivity was also graded according to the percent of invasive or intraductal breast tissue that expressed nm23.

In the patient population studied, only 16 of the 40 specimens analyzed contained an intraductal component, whereas 40 of the patients analyzed contained an invasive component. Thus, our analyses focused on the invasive component only. Tissue with 40 percent or less positivity was considered negative for nm23. The 40 percent cutoff value was derived from the literature (24) and the patient distribution which was roughly divided in half. Figure 2

shows two different breast cancer tissues and their respective negative controls. Panel A is an example of a breast tissue that was graded as positive (100%) for nm23 expression and Panel C was graded as nm23 negative since less than 40% of the invasive tissue was stained for nm23. Panels B and D are the negative controls for A and C, respectively. In the intraductal carcinoma tissues evaluated, 14 of the 16 (88%) specimens were positive for nm23 staining. In the two intraductal tissues negative for nm23 staining, only one was in the recurrent patient group. Among the invasive tumors evaluated, 21 of the 40 (53%) were negative for nm23 staining, of these, 12 were recurrent and 9 were non-recurrent patients. The nm23 staining in all breast samples analyzed to date has been cytoplasmic.

The Kaplan-Meier disease-free survival curve as a function of nm23 positivity is shown in Figure 3. The degree of nm23 positivity (<40% vs ≥40%) did not offer a selective advantage against recurrence or survival, (Table 3) as would be predicted by the hypothesis that nm23 has anti-metastatic properties. Additionally, there was no statistically significant difference between nm23 positive (≥40% positive) and negative (<40% positive) populations with respect to the prognostic markers analyzed (Table 3)

DISCUSSION

Our experience in the analyses of breast cancer patient specimens, tissue culture cell lines and cell lysates from primary human breast cancer xenografts, by both immunohistochemical and western blot analysis has shown that in some cases one procedure may be superior to the other. With human tumor cell lines grown in athymic nude mice models, the western blot technique provides superior information compared to immunohistochemistry procedures since there is a high level of nonspecific tissue reactivity of mouse-derived tumors with certain detection antibodies. These tissues are easily analyzed for nm23-H1 and nm23-H2 proteins using western blot techniques (Table 1). Immunohistochemistry may be more valuable in the analyses of human breast carcinoma, particularly in patients with very small tumor size where extracts may not be available.

We have shown that nm23-H1 levels vary considerably between cell lines, xenografts and breast cancer patients. Our data indicated that MCF-7 cells can metatasize to the lung if careful histological analyses is performed. This observation is in agreement with Shafie *et al* (25). Our data suggested a trend (R=-0.51) for lower nm23-H1 levels in both the xenografts and the cell lines that had high metastatic potential in athymic nude mice, as compared to their non-metastatic counterparts. More cell lines and xenografts need to be analyzed to prove whether this is a significant observation.

The analyses of the relationship between disease recurrence, overall survival and the levels of nm23 as measured by immunohistochemistry in the node negative breast cancer population was not significant. The limitations of a retrospective study and the small number of recurrent and non-recurrent patients may be responsible for the lack of correlation between nm23 levels and the risk of disease recurrence. These two groups, however, were evenly matched for

various clinical factors that might relate to disease recurrence. The purpose of matching these patients was to determine whether nm23 was an independent prognostic indicator. Based on the observations in this study, nm23 may not be an independent indicator of disease progression or aggressiveness.

Although several human breast cancer studies have shown an association between nm23 levels and patient survival and/or prognosis (6-8), this is the first attempt to control for all other prognostic factors that may obscure the true role of nm23 expression in patient survival. In particular, the association of low nm23 expression and lymph node involvement has proven significant in different studies (2,6-7,9). Since this study selected only node negative breast cancer patients, perhaps this is a reason for the lack of correlation of disease recurrence with nm23 positivity. Since lymph node involvement at the time of diagnosis is known to be associated with a poor prognosis, the next stage of analysis must be the predictive ability of nm23 levels for disease recurrence and survival in node negative patients. From the purely clinical perspective, the usefulness of nm23 as a prognostic marker must be proven in the unknown risk group (node negative at diagnosis). In this matched retrospective node negative breast cancer population, nm23 levels were not predictive of either disease recurrence or overall survival. It is possible that there are potentially recurrent patients still undetected in the non-recurrent data set. The probability of this was reduced by extending the follow-up time in the non-recurrent patients; however, the range for the time to recurrence was between 0.7 and 9.2 years so there may be more undetected recurrences in the non-recurrent group (Table 3).

The xenograft and cell line data support the possibility that nm23 levels may be related to metastatic potential but only more extensive clinical studies will clarify the potential for nm23 as a predictor of clinical outcome in node negative breast cancer.

ACKNOWLEDGMENTS

The technical assistance provided by Sandy Johnston, Rena Long, and Nora Zbieranski, was invaluable and greatly appreciated. This project was sponsored in part by the Department of the Army (DAMD17-94-J-4342). We would also like to thank Dr. Patricia Steeg and Oncologix™ for the antibodies used in this study.

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TABLE 1

EXPRESSION OF nm23-H1 IN BREAST CANCER CELL LINES, AND XENOGRAFTS DERIVED FROM PRIMARY HUMAN TUMORS

EXPRESSION OF nm23-H1 (ng/μg of protein)				METASTATIC POTENTIAL	
CELL TYPE	**N**	**MEAN (± SE)**		**METS/ANIMAL**	**(%)**
MCF-7	6	1.642	(0.137)	2/6	(33)
MDA-MB-231	7	0.173	(0.047)	5/7	(71)
MDA-MB-435	5	0.369	(0.118)	5/10	(50)
XENOGRAFT ¶	8				
UISO-BCA-1	8	0.542	(0.076)	0/50	(0)
UISO-BCA-4	8	0.429	(0.172)	0/50	(0)
OSHTMAM-4	7	0.605	(0.086)	1/5	(20)
MAX-F-401	6	0.388	(0.047)	3/8	(38)
MAX-583	8	0.291	(0.074)	1/5	(20)
MAX-713	7	0.182	(0.069)	1/5	(20)
MAX-713 (mtg)§	8	0.137	(0.039)	1/5	(20)
GI-101	9	0.258	(0.038)	3/5	(60)

¶ Human breast cancer specimens were transferred to nude mice. The resulting tumor, grown in nude mice was analyzed for nm23 -H1 by western blot.
§ Xenograft was injected in matrigel (mtg).

TABLE 2

FACTOR	NON-RECURRENT mean ± SE	N=19 (%)	RECURRENT mean ± SE	N=21 (%)
AGE	58.7 ± 15.8		62.9 ± 14.7	
CATHEPSIN D	54.1 ± 18.5		60.8 ± 29.4	
TUMOR SIZE	1.9 ± 0.9		2.0 ± 1.0	
% S PHASE [a]	7.3 ± 5.6		6.9 ± 3.7	
% TUMOR [b]	41.0 ± 25.2		53.2 ± 25.9	
% ANEUPLOID		67		56
% ER POSITIVE		33		67
% PR POSITIVE		35		65
TUMOR GRADE[c] (#)				
1	0		1	
2	7		6	
3	8		11	
TIME TO RELAPSE/ OR FOLLOW-UP (years)				
RANGE	3.3-13.8		0.7-9.2	
MEDIAN	5.1		2.7	
MEAN	6.3		2.9	

[a] The non-recurrent group contains 12 patients and the recurrent group contains 15 patients for % S phase analysis by flow cytometry.

[b] All slides used in this study contain sufficient tumor for analyses. The mean % tumor ± the SE of the tumors are represented here.

[c] Tumor grade was available for 15 non-recurrent and 18 recurrent patients.

TABLE 3

FACTOR	**nm23 < 40 N = 21** mean ±SE (#)	**nm23 ≥ 40 N = 19** mean ±SE (#)	**p-value significance**
AGE	61.0 ± 13.20	61.0 ± 17.50	0.860
CATHEPSIN D	51.9 ± 22.39	63.9 ± 26.26	0.098
TUMOR SIZE	1.9 ± 0.88	2.0 ± 1.03	0.684
% S PHASE [a]	7.0 ± 5.20	7.2 ± 3.88	0.806
% TUMOR [b]	48.6 ± 24.91	47.8 ± 26.26	0.684
# ANEUPLOID			
no (DNA=1.0)	7	6	1.000
yes (DNA>1.0)	12	8	
# ER POSITIVE			
>10	11	10	1.000
≤ 10	10	9	
# PR POSITIVE			
> 10	8	9	0.750
≤ 10	13	10	
TUMOR GRADE[c]			
1	0	1	0.424
2	8	5	
3	9	10	
OUTCOME	**(#) (%)**	**(#) (%)**	
# of recurrences	12 (57)	9 (47)	0.752
# of deaths	5 (24)	7 (37)	0.494

a The non-recurrent group contains 12 patients and the recurrent group contains 15 patients for % S phase analysis by flow cytometry.

b All slides analyzed contained a sufficient tumor component. The % of tumor per slide analyzed is represented here.

c Tumor grade was available for 15 non-recurrent and 18 recurrent patients.

LEGENDS

FIGURE 1: Western blot analysis of human breast cancer cell lines and xenografts. (A) The western blot of the breast cancer cell lines MDA-MB-435 and MCF-7 is shown. The standard curve contained 10, 20, 40, 80 and 160 ng of purified nm23-H1 protein in lanes 2-6, respectively. Lanes 8 and 10 contain cell pellet extracts from MDA-MB-435 and MCF-7 cell lines, respectively. Lanes 1, 7 and 9 are blank. **(B)** Xenografts from human breast tumors, grown in nude mice were analyzed by western blot for nm23 concentrations. Lanes 1-5 represent known nm23-H1 standards and contain 100, 75, 50, 30 and 20 ng of purified protein, respectively. Lane 6-12 represent nm23 levels in xenografts UISO-BCA-1, UISO-BCA-4, MAXF-401, MAXF-583, MAXF-713 (with matrigel), MAXF-713, OHSTMAM-4 and GI101 as described in Methods. Lanes 6, and 7 are non-metastatic in athymic nude mice and lanes 8- 13 are metastatic tumors. The upper bands represent nm23-H1 and the lower bands in the tumor extracts are nm23-H2. All nm23-H1 values for each tumor were calculated from the standard curve shown.

FIGURE 2: Immunohistochemical analyses of nm23 in human breast tumors. Human breast cancer tissues were stained for nm23 using polyclonal anti-nm23 antibody as described in Methods. Panel A represents an invasive breast carcinoma staining positive for nm23 (100% of the tissue expresses nm23). Panel B is the negative control for the same tissue. Panel C is an invasive breast tissue that was graded as nm23 negative since less than 40% of the tissue stained for nm23 and panel D is the negative control for this tissue.

FIGURE 3: Relationship between nm23 positivity and disease-free survival in node negative breast cancer patients. The nm23 analyses by immunohistochemistry in the node negative breast cancer study was plotted as a function of relapse-free survival using the method of Kaplan and Meier (20). The patients with negative nm23 staining intensity are identified as the solid line (N=21 with 12 relapse events). Those patients that were nm23 positive were identified with a dashed line (N=19 with 9 relapse events). No prognostic advantage was observed in patients who had nm23 positive tumors.

Figure 1

1a

1b

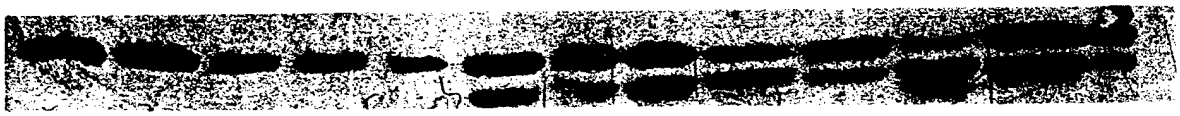


Figure 2

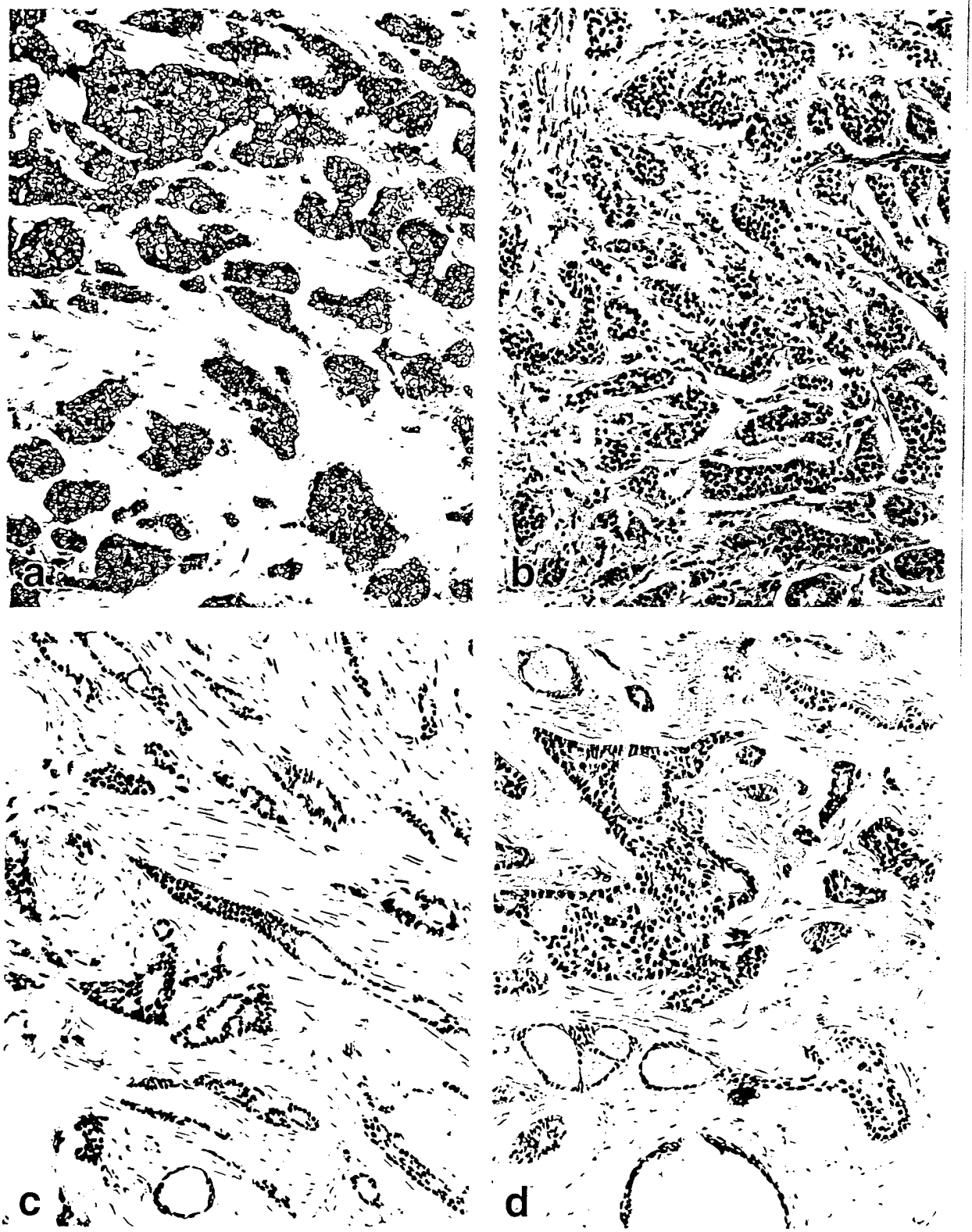
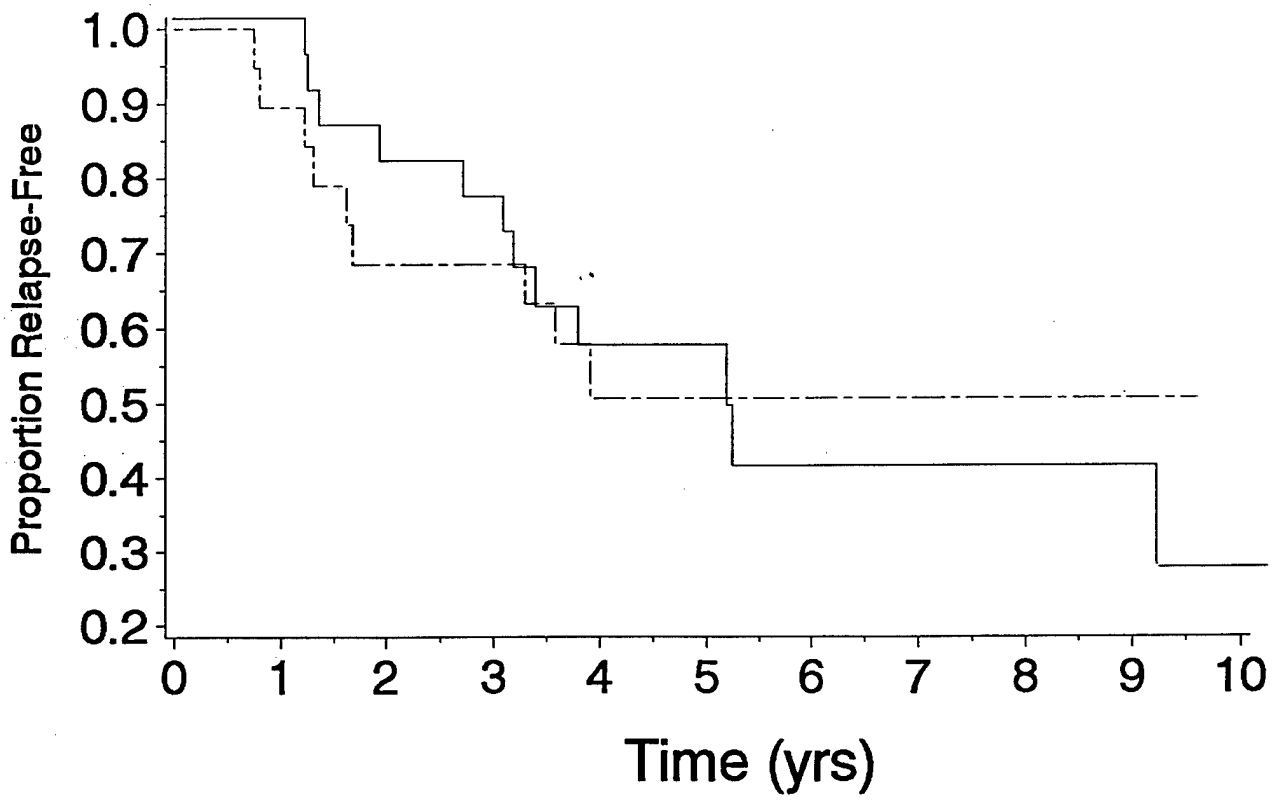


Figure 3


Proportion Relapse-Free
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
1
2
3
4
5
6
7
8
9
10
Time (yrs)